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Real-Time PCR for Quantification of Human Herpesvirus 6 DNA from Lymph Nodes and Saliva

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A real-time quantitative PCR assay has been developed to measure human herpesvirus 6 (HHV-6) DNA in biological specimens. The assay sensitivity was 10 copies of DNA per well, with a linear dynamic range of 10 to 10^7 copies of HHV-6 DNA. Intra- and interassay variations were, respectively, 0.88 and 0.8% for samples containing 10^2 DNA copies, 0.99 and 0.96% for samples containing 10^4 copies, and 0.76 and 0.9% for samples containing 10^6 copies. Among 34 saliva samples from healthy subjects, 26 were found to contain HHV-6 DNA (76.5%; median, 23,870 copies/ml), and following a single freeze-thaw cycle, 25 of the same samples were found to be positive for HHV-6 DNA, although at a statistically significantly lower concentration (median, 3,497 copies/ml). The assay enabled detection of HHV-6 DNA in lymph node biopsies from patients with Hodgkin's disease (HD) (13 of 37 patients [35.1%]), B-cell neoplasms (8 of 36 patients [22.2%]), and T- or NK-cell neoplasms (3 of 13 patients [23.1%]), with concentrations ranging from 100 to 864,640 HHV-6 copies per μg of DNA (HHV-6B being found in every case except two). All HD patients infected with HHV-6 presented clinically with the nodular sclerosis subtype of HD. The real-time quantitative PCR assay developed here was simple to perform and was sensitive over a wide range of HHV-6 concentrations. It therefore appears to be of potential value in clinical investigation or diagnosis of HHV-6 infection.

Human herpesvirus 6 (HHV-6) was first identified in 1986 (37), and presently two types (A and B) are known (1). HHV-6 is prevalent throughout the world, and most children acquire at least one of the two types of this betaherpesvirus when under they are 2 years old (24, 29), with primary infection usually occurring between 6 and 24 months of age. HHV-6B has been identified as the cause of exanthem subitum (roseola), a typically mild eruptive childhood disease (44) that is occasionally complicated by development of meningitis (20), meningoencephalitis (3), or hepatitis (4). When primary infection occurs later in life, in teenagers or in adults, the presence of the virus has been associated with hepatitis, encephalitis, and a mononucleosis syndrome (2). By contrast, infection with HHV-6A is generally symptomless and appears to be unrelated to any specific pathology (8). Following primary infection, HHV-6A and HHV-6B are thought to persist for life as a latent form in mononuclear cells, macrophages, and vascular endothelial cells and as a low-level chronic replicating form located in oropharyngeal epithelial cells (17). HHV-6 DNA can normally be detected in saliva, which as a result is considered to be the most likely vector for HHV-6 transmission (18, 22, 25, 36). As observed with cytomegalovirus (another betaherpesvirus), HHV-6 can reactivate in individuals whose immune systems have been compromised by disease (e.g., AIDS) or by treatment (e.g., with posttransplantation immunosuppressive drugs) and may therefore be considered an opportunistic virus.

Evidence suggests a potential association between HHV-6 and lymphoproliferative diseases. (i) HHV-6 was initially isolated from the blood of patients with lymphoproliferative disorders. (ii) Serological studies show significantly higher anti-HHV-6 titers in Hodgkin's disease (HD) patients than in healthy controls (27, 41). (iii) HHV-6 DNA has been found in lymph nodes from 29 to 73% of patients suffering from lymphoproliferative disorders (5, 7, 11, 42), with these figures varying according to the number of patients studied, stage of human immunodeficiency virus infection, and assay technique employed (PCR or in situ hybridization). (iv) A latently HHV-6-infected cell line was recently established from pathological tissue derived from a case of Burkitt's lymphoma (6) that was HHV-6 positive and Epstein-Barr virus negative.

Quantitation of HHV-6 in lymph nodes from patients with lymphoproliferative disorders would be of use in assessing the pathological role of this virus in the clinical development of the disease. We have therefore designed a sensitive and specific real-time PCR assay for quantitative analysis of HHV-6A or HHV-6B DNA. The assay was initially used to quantify HHV-6 DNA in saliva samples from healthy subjects before being applied to detection of the virus in lymph node biopsies from patients with lymphoproliferative diseases. Prior to use in the assay, lymph node samples had been stored frozen. We therefore also evaluated the effect of freezing and thawing cycles on quantitation of HHV-6 DNA.

MATERIALS AND METHODS

Patients and samples. Lymph node biopsies from 86 human immunodeficiency virus-seronegative patients with lymphoproliferative disorders were retrospectively included in this study. The patients had all been initially hospitalized in the department of Clinical Haematology at Limoges University Teaching Hospital in France, and disorders were diagnosed on the basis of histopathological analysis of lymph node biopsies. Aliquots of each specimen were frozen at −80°C immediately after biopsy and then maintained at this temperature until used in the assay. Patients were divided into three groups: (i) 37 patients with HD (group 1), (ii) 36 patients with B-cell neoplasms (group 2), and (iii) 13 patients with T- or NK-cell neoplasms (group 3). Table 1 shows mean age and sex ratio data plus further details of patient groupings carried out according to World

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TABLE 1. Patient groups

Group	Diagnosis	No. of patients	Mean age (yr)	No. male/no. female
1 (HD, classical)	Nodular sclerosis Hodgkin's lymphoma	31	39.2	16/15
	Mixed-cellularity Hodgkin's lymphoma	4	70.2	2/2
	Lymphocyte-depleted Hodgkin's lymphoma	2	55	1/1
	Total	37	43.4	19/18
2 (B-cell neoplasms)	B-cell chronic lymphocytic leukemia	1	70	1/0
	Lymphoplasmacytic lymphoma	2	67	1/1
	Splenic marginal zone B-cell lymphoma	2	62.5	0/2
	MALT-type lymphoma	2	68.5	0/2
	Follicular lymphoma	14	55.4	7/7
	Mantle cell lymphoma	2	70	1/1
	Diffuse large B-cell lymphoma	13	67.5	6/7
	Total	36	64.7	16/20
3 (T- and NK-cell neoplasms)	Precursor T-lymphoblastic lymphoma	1	12	1/0
	Mycosis fungoides	1	63	1/0
	Peripheral T-cell lymphoma	4	72.7	3/1
	Angioimmunoblastic T-cell lymphoma	5	75.8	4/1
	Anaplastic large-cell lymphoma	2	42	2/0
	Total	13	63.8	11/2

Health Organization classification guidelines for neoplastic diseases of lymphoid tissues (19). Saliva samples were also collected from 34 healthy adults and maintained at 4°C until use in the assay.

HHV-6 strains. HHV-6A (GS strain) cultured in HSB-2 cells was kindly donated by H. Agut, La Pitié Salpêtrière, Paris, France. HHV-6B (Z29 strain; no. VR-1467) cultured in Molt-3 cells was obtained from the American Type Culture Collection (Manassas, Va.).

Preparation of DNA. Shredded fragments of lymph node biopsies were washed in sterile phosphate buffer solution and left to stand at 4°C in a homogenization buffer (0.25 M saccharose, 25 mM Tris-HCl [pH 7.5], 25 mM NaCl, 25 mM $MgCl_2$) until completely disintegrated. Lysis took place overnight in Tris-EDTA buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA [pH 8], 10 mM NaCl) containing 0.5% sodium dodecyl sulfate and 250 μg of proteinase K per ml. Finally, standard phenol-chloroform procedures were used to extract DNA, which was then quantitated by spectrophotometric measurement at 260 nm prior to freezing at −80°C and storage until required.

DNA in saliva was extracted from 1-ml samples using the QIAamp blood kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. Extracts were then resuspended in 100 μl of buffer, and aliquots were assayed either immediately or following a freeze-thaw cycle at −80°C.

Oligonucleotide primers and TaqMan probe. By using the OMIGA software program version 1.1.3 (Oxford Molecular, Oxford, United Kingdom), published sequences of the HHV-6A (16) and HHV-6B (12, 21) genomes were compared for mismatches before selection of a highly conserved region (U22 gene) specific to HHV-6 and therefore distinct from any other herpesvirus (14). The primers and probe for the real-time quantitative PCR assay were derived from published data on the U22 open reading frame of HHV-6A strain U1102 and designed using Primer Express Software (Applied Biosystems, Courtaboeuf, France). The forward primer and reverse primer were, respectively, 397F (5′-TCG AAA TAA GCA TTA ATA GGC ACA CT-3′) and 493R (5′-CGG AGT TAA GGC ATT GGT TGA-3′), both of which were designed to amplify a target 99-bp fragment from the HHV-6A and HHV-6B U22 genes in human samples. The TaqMan probe (435T) was labeled with 6-carboxyfluorescein at the 5′ end as the reporter dye and with 6-carboxytetramethylrhodamine at the 3′ end as the quencher (FAM-CCA AGC AGT TCC GTT TCT CTG AGC CA-TAMRA). An A-to-G substitution in the sequence of HHV-6B at position 11 of the reverse primer and an AC-to-GT substitution of the probe at positions 13 and 14 were accepted for the primer-probe design. The primers and the TaqMan probe were purchased from Isoprim (Toulouse, France) and Perkin-Elmer Biosystems (TIB MOLBIOL, Berlin, Germany), respectively. An extensive search of several databases, including the EMBL and GenBank databases, indicated that neither the primers nor the probe shared significant homology with any known nucleotide sequences.

TaqMan PCR. All samples were checked for the absence of inhibitors by amplification of the DQA1 gene of the major histocompatibility complex system prior to quantitation of HHV-6. Each 50-μl PCR mixture contained 100 ng of purified DNA from lymph node extracts or 10 μl of DNA from saliva samples, a 900 nM concentration of each primer, a 100 nM concentration of the probe, and 25 μl of TaqMan Universal PCR master mix (Applied Biosystems). The PCR mixtures in 96-well microtiter plates were first incubated at 50°C for 2 min to activate the uracil *N*′-glycosylase and then incubated at 95°C for 10 min. to inactivate the uracil *N*′-glycosylase and activate AmpliTaq Gold (Applied Biosystems), followed by 50 two-step cycles at 95°C for 10 s and 60°C for 1 min, using an ABI PRISM 7700 sequence detection system (Applied Biosystems). Amplified products were determined by continuous monitoring of fluorescence. After data collection, the cycle threshold (Ct) number was calculated by determining the point at which the fluorescence exceeded an arbitrary lower limit, chosen to cover the range of readings given by all standards in the exponential phase; the Ct value therefore reflected the overall quantity of target copies in samples. Each specimen was run in triplicate and considered positive only if at least two of the three results exceeded the threshold. Serial dilutions of plasmid DNA as a standard curve (1 copy to 10^6 copies) for quantitative analysis, as well as positive and negative controls, including a reaction mixture without DNA, were included in every run and tested in triplicate. Sequence Detector software version 1.6.3 (Perkin-Elmer Biosystems) was used to determine standard curves to calculate the precise quantities of target templates in the samples.

Standardization. Two plasmids, each containing one copy of the U22 target sequence from HHV-6A and HHV-6B, respectively, were used as standard controls for HHV-6 quantitation. They were made using DNAs from HHV-6A (GS strain) and HHV-6B (Z29 strain), amplified with primers 397F and 493R, and cloned into the PCR 2.1 TA cloning vector (Invitrogen BV, Luel, The Netherlands). The resulting standard controls were fully sequenced before the plasmids were expanded. The concentration of purified plasmid DNA was determined by spectroscopy at 260 nm, and the corresponding copy number was calculated. Determination of standard curves for both plasmids was based on triplicate tests over a range of 10 to 10^8 copies per well, with identical results. Further studies included a single plasmid (U22 HHV-6B) tested in triplicate over the same range.

Determination of HHV-6 subtype. A nested PCR was used to amplify a 492-bp fragment of the U31 gene. This fragment included a *Hin*dIII restriction site for HHV-6 type B, which is absent in type A. Primers employed for the initial PCR step were H6/594 (5′-TTA ACG GTC GCG TTC TAA CC-3′) and H6/1809 (5′-ACG CCT CGT TGA ATA CTT CG-3′), amplifying a 1,216-bp fragment. Subsequent nested PCR utilized primers H6/772 (5′-AAG AAG GCT ATC ACT TAG ACA CGG-3′) and H6/1263 (5′-TTA GGA TAA GAA GCT CGG CG-

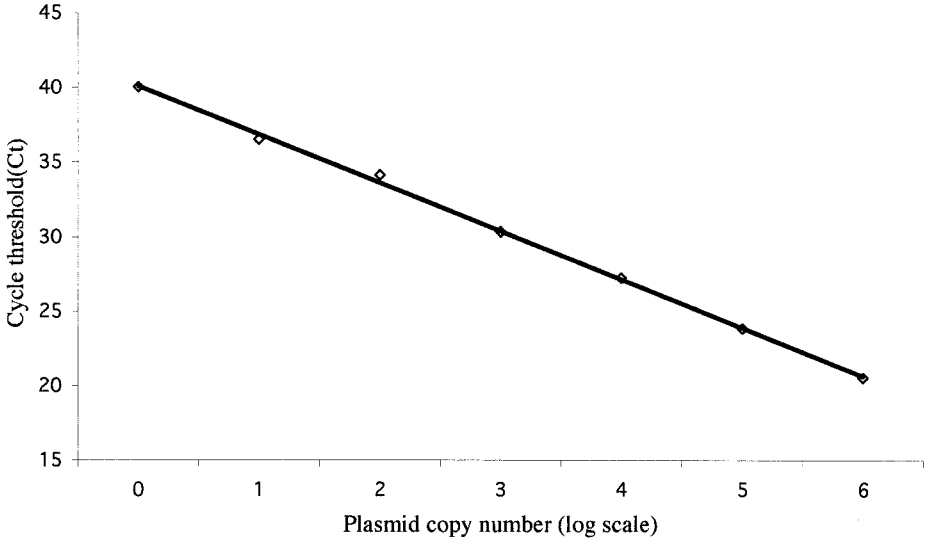

FIG. 1. Standard curve obtained for HHV-6 quantification. Plasmid PCRII containing one copy of the U22 target sequence was used to construct the standard curve for HHV-6 quantification. Tenfold serial dilutions ranging from 10^7 copies to 1 copy of plasmid were tested in triplicate, and the mean Ct values were plotted against copy number (correlation coefficient, 0.9986; slope, −3.375).

3′). Samples in the first PCR step were 500 ng of DNA from lymph node extracts or 10 μl of DNA from saliva samples to make up 50 μl of reaction mixture containing 10 mM Tris (pH 8.6), 500 nM $MgCl_2$, 50 mM KCl, a 300 mM concentration of each deoxynucleoside triphosphate, a 0.6 μM concentration of each primer, and 1.5 U of *Taq* polymerase (Qiagen). Subsequent nested PCR employed a 50-μl reaction mixture composed of 1 μl of product obtained from the first PCR, 10 mM Tris (pH 8.6), 200 nM $MgCl_2$, 50 mM KCl, a 250 mM concentration of each deoxynucleoside triphosphate, a 1 μM concentration of each primer, and 1.5 U of *Taq* polymerase. The two amplification procedures were conducted under identical conditions: 94°C for 6 min; then 94°C for 30 s, 53°C for 30 s, and 72°C for 45 s for 40 cycles; and finally 72°C for 7 min. Amplified products were then incubated for 1 h at 37°C with *Hin*dIII enzyme (Roche, Meylan, France) before analysis by gel electrophoresis; the restriction site produced 370- and 122-bp fragments.

Statistical analysis. The proportions of positive values obtained for each group of patients were compared using Fisher's exact test. Median values were calculated for each group, and results were analyzed by the Kruskal-Wallis test. Results for saliva samples before and after a thaw cycle were compared using the Wilcoxon matched-pairs test.

RESULTS

Optimization of TaqMan conditions. The first stage in this study was to obtain reproducible detection of DNA extracted from the GS strain of HHV-6A and serially diluted plasmid controls, using standard PCR conditions recommended by Applied Biosystems. The second stage involved optimizing the reaction conditions by varying the primer and probe concentrations to obtain optimal fluorescent signal accumulation and rapid measurement of Ct values. Optimization was carried out by methodical variation of each test parameter under standard PCR conditions. The primer and probe concentrations tested ranged from 50 to 900 nM for primers 397F and 493R and from 100 to 200 nM for the 435T probe.

All of these trials were carried out with dilutions of plasmid controls. Optimum amplification profiles were obtained with a primer concentration of 900 nM in conjunction with Mg^{2+} at a concentration of 2.5 mM and a probe concentration of 100 nM. Several DNA concentrations, i.e., from 50 ng to 1 μg of DNA in a final volume of 50 μl, were also tested. Optimum reproducible results were obtained for 100 ng of DNA extract. Two different final reaction volumes, 25 and 50 μl, were also tested; the latter proved to be the most valid. The annealing extension temperature and number of cycles were also determined experimentally. The best results were obtained with a temperature of 60°C for 50 cycles.

Reference curves. The 99-bp fragments obtained by amplification of HHV-6 DNAs extracted from GS strain HHV-6A and from Z29 strain HHV-6B by using primers 397F and 493R were cloned into the PCR2.1-TOPO plasmid, thus providing a reproducible source of standard DNA. The two plasmids were quantitated by UV spectroscopy. The same assay was applied to both the A and B strains. DNA dilution started at 10^{11} copies per μl and extended through three sets of 10-fold dilutions to give 1 copy per μl. The three series of samples were then amplified (from 1 to 10^7 per well) in the same run, and the data collected were used to generate a linear-log regression plot (Fig. 1). Similar results were obtained for the two standard plasmids. The U22 plasmid containing the HHV-6B gene was further used as a standard control for each run.

Sensitivity of PCR. Serial half-log dilutions of the plasmid standards, ranging from 10^7 copies to 1 copy per well, were made to determine the linearity, precision, and sensitivity of the TaqMan assay. Ct values measured in triplicate, (*y* axis) were plotted against the log of the input HHV-6 copy number (*x* axis) (Fig. 1), producing a linear slope. The calculated coefficients of variation (CVs) were less than 5% for ≥10 copies per well and 7.2% for 1 copy per well. The assay sensitivity was therefore estimated as 10 copies per well.

Specificity of PCR. DNAs from the following other human herpesviruses were tested using the assay: Epstein-Barr virus strain P3HR-1, kindly donated by J. Icard, Toulouse, France; cytomegalovirus strain AD 169 and HHV-8 strain BC3, purchased from the American Type Culture Collection; and herpes simplex viruses types 1 and 2, varicella-zoster virus, and

TABLE 2. Intra- and interassay variabilities

No. of copies	Intra-assay variability			Inter-assay variability		
	Ct		CV (%)	Ct		CV (%)
	Mean	SD		Mean	SD	
10^6	19.82	0.15	0.76	20.03	0.16	0.8
10^4	26.33	0.26	0.99	26.02	0.25	0.96
10^2	32.97	0.29	0.88	33.12	0.3	0.9

HHV-7, isolated from patients. As expected, no cross-reactivity was obtained with any of the human herpesviruses tested.

Assay precision. Intra-assay variability was determined by measuring the Ct values of low (100 copies per well)-, middle (10^4 copies per well)-, and high (10^6 copies per well)-copy-number plasmid controls, with 10 replicates per batch. Interassay variability was tested by running the same controls with 10 replicates on three consecutive days. The results obtained are summarized in Table 2.

Quantitation of the HHV-6 genome in saliva from healthy adults. As expected, a high number of DNA samples extracted from saliva were found positive for HHV-6: 26 of 34 (76.5%) for DNA extracted from unfrozen samples and 25 of 34 (73.5%) for DNA subjected to a freeze-thaw cycle. HHV-6 DNA in unfrozen extracts ranged in quantity from 2,240 to 347,328 copies per ml of saliva (Fig. 2). The majority of the samples contained between 10^4 and 10^5 copies/ml, with a median value of 23,870. The results obtained with DNA samples subjected to a freeze-thaw cycle showed statistically significantly ($P < 0.0001$) decreased values in all cases, with a median of 3,497.

Quantitation of HHV-6 DNA in lymph nodes of patients. HHV-6 genome sequences were detected in 13 of 37 (35.1%), 8 of 36 (22.2%), and 3 of 13 (23.1%) lymph node biopsies from patients in groups 1, 2, and 3, respectively (Table 3). The mean age of HHV-6-positive patients in group 1 was 34.8 years. For HD patients, HHV-6 DNA in positive lymph nodes ranged from 100 to 864,640 copies per μg of DNA (Fig. 2), whereas for non-Hodgkin's lymphoma (NHL) patients, ranges were less extensive (100 to 44,290 DNA copies in group 2 [B-cell lymphomas] and 750 to 33,800 copies in group 3 [T- or NK-cell lymphomas]). No statistically significant difference in the proportions of positive values and their spreads was detected between the three groups.

HHV-6 types. All HHV-6-positive saliva samples were identified as HHV-6B strains. Lymph node DNA was also identified as belonging to the HHV-6B strain in all but two cases (22 of 24 [91.7%]), one in group 1 and one in group 2 (Table 3).

DISCUSSION

The real-time quantitative PCR assay developed here to determine the number of HHV-6 genome copies in saliva appears to be sensitive, precise, and rapid. Quantitation of HHV-6 DNA can be obtained in under 2 h, and the method is less laborious than traditional PCR techniques (38). Sensitivity is high, with as few as 10 HHV-6 DNA copies detected per well, thus reflecting sensitivity levels in previous studies (34,

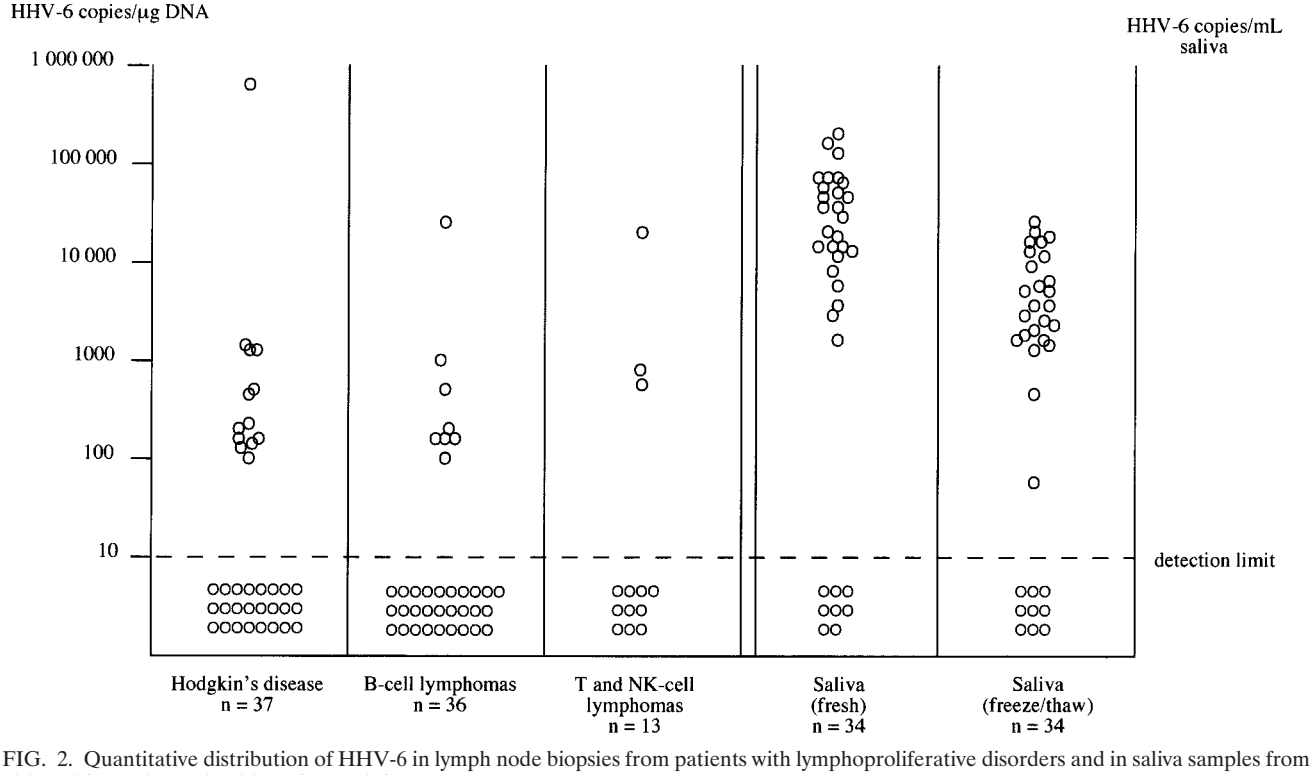

FIG. 2. Quantitative distribution of HHV-6 in lymph node biopsies from patients with lymphoproliferative disorders and in saliva samples from healthy subjects, determined by using real-time PCR assay.

TABLE 3. HHV-6 genome quantification and type according to pathology

Group	Diagnosis	No. of patients	No. (%) of HHV-6 positive patients	HHV-6 type	Mean copy no.
1 (HD, classical)	Nodular sclerosis Hodgkin's lymphoma	31	13 (41.9)	12B/1A	6,711.4
	Mixed-cellularity Hodgkin's lymphoma	4	0		
	Lymphocyte-depleted Hodgkin's lymphoma	2	0		
	Total	37	13 (35.1)		
2 (B-cell neoplasms)	B-cell chronic lymphocytic leukemia	1	0		
	Lymphoplasmacytic lymphoma	2	0		
	Splenic marginal zone B-cell lymphoma	2	1 (50)	B	18
	MALT-type lymphoma	2	0		
	Follicular lymphoma	14	3 (23.1)	3B	39
	Mantle cell lymphoma	2	0		
	Diffuse large B-cell lymphoma	13	4 (30.8)	3B/1A	1,140.7
	Total	36	8 (22.2)		
3 (T- and NK-cell neoplasms)	Precursor T-lymphoblastic lymphoma	1	0		
	Mycosis fungoides	1	0		
	Peripheral T-cell lymphoma	4	1 (25)	B	3,380
	Angioimmunoblastic T-cell lymphoma	5	1 (20)	B	75
	Anaplastic large-cell lymphoma	2	1 (50)	B	91
	Total	13	3 (23.1)		

35); two samples in our study were detected at this low level. A wide linear range (from 10 to 10^8 copies) enables reliable detection of from low to extremely high numbers of HHV-6 copies; the present study recorded one clinical sample with 86,464 copies per well. The assay therefore may be said to provide reproducible quantitative results over a wide range of concentrations. Moreover, the same levels of sensitivity and reproducibility are obtained with both types of HHV-6, consequently eliminating the need to ascertain the HHV-6 type before proceeding with the assay. Intra-assay variations of 0.88, 0.99, and 0.76% were recorded for samples containing, respectively, 10^2, 10^4, and 10^6 copies per well, with interassay variations for the same concentration range being 0.8, 0.96, and 0.9%, respectively. Both inter- and intra-assay variations were low compared with those from previously published assays (26, 30–32) and consequently are well within the limits of acceptance for clinical use. The risk of false-positive results due to contamination from previously amplified products is greatly reduced since the assay is performed in a single step, and the method for prevention of enzymatic contamination (use of dUTP instead of dTTP in the reagent mixture and use of the uracil *N′*-glycosylase enzyme) further increases reliability of the assay (23). All sample controls in our study proved negative, thus confirming the absence of contamination in every assay batch.

Application of the TaqMan assay to quantitative measurement of HHV-6 DNA in biological samples was carried out by evaluating HHV-6 copy numbers in 34 saliva extracts from healthy subjects. The virus was found in 76.5% of this normal population, which is similar to previously published prevalence values (9, 15). Viral loads for positive samples ranged between 2,240 and 347,328 copies per ml for fresh extracts. However a sharp decrease in amplification efficiency was noted when DNA extracts from saliva had been frozen prior to testing. Positive samples after single freeze-thaw cycles gave values ranging from 74 to 39,923 copies per ml (Fig. 2), a statistically significant drop ($P < 0.0001$) compared to values for fresh DNA extracts.

Several studies have raised questions regarding a possible association between HHV-6 presence in lymph nodes and the development of lymphoproliferative diseases. Our present large-scale study, using sensitive PCR analysis of 100 ng of lymph node tissue DNA, detected the HHV-6 genome in 35.1% of all HD patients. Minor differences between our results and those reported by others (10, 40, 43) probably reflect variations in the sensitivities of the various tests employed and in the amounts of DNA analyzed, as well as differences in the populations studied. By contrast, however, Shiramizu et al. (39) found no HHV-6 sequences in a cohort of 47 pediatric HD patients. It is interesting that all of the HHV-6-positive HD samples detected in our study came from patients suffering from the nodular sclerosis form of HD; histological analysis to find other HD subtypes proved negative (Table 3).

The prevalence of HHV-6 DNA among NHL patients was similar to that observed for HD patients (22.2% for B-cell neoplasms and 23.1% for T- and NK-cell neoplasms). Few studies on the presence of HHV-6 in the lymph nodes of NHL patients are presently available; some teams report no HHV-6 in lymph nodes (10, 41), while others have recorded values close to those obtained for HD (13, 43). Although HHV-6 displays tropism for T lymphocytes, we found HHV-6 DNA in eight cases of B-cell neoplasms. Interestingly, our assay detected HHV-6 sequences in one case of angioimmunoblastic T-cell lymphoma among five studied, thus confirming suspicions of the presence of this lymphotropic virus in this clinical

condition (28). HHV-6 was also found in two other T-cell neoplasms (Table 3).

The present study confirmed previous observations (10, 43) that HHV-6B is more common than HHV-6A: 91.7% (22 of 24) versus 8.3% (2 of 24) (Table 3). This result appears not to be linked to any clinical or pathological parameters.

Very few previous studies have reported attempts to quantify HHV-6 genomes in lymph nodes. Valente et al. (43) adopted an indirect approach combining two tests with different sensitivities, i.e., PCR and Southern blot analysis, thus providing an assay discriminating between low and high levels of viral DNA in samples. The assay gave negative results for lymph node samples from 52 HD patients, with levels of <10,000 HHV-6 copies per μg of DNA (the sensitivity limit of their Southern blot was 10,000 copies per μg of DNA). Similar results were obtained by Di Luca et al. (10). To our knowledge, the only report of precise HHV-6 quantitation in lymph nodes from lymphomas was by Ohyashiki et al. (33), using a PCR-enzyme-linked immunosorbent assay. The values that they observed for positive samples ranged between 6.4 copies per μg of DNA for a B-cell lymphoma and 3,705 copies per μg of DNA in the case of an immunoblastic lymphadenopathy.

The values obtained in the present study are quite high, particularly if we take into account the fact that each specimen was frozen twice (before and just after DNA extraction). This probably reflects the high sensitivity of the test employed. Surprisingly, in each group, one specimen showed a higher number of copies than the others; this fact was more pronounced in group 1 (HD), where as many as 864,640 copies per μg of DNA were observed in the lymph node of one patient. Although the lack of prevalence differences observed in the three groups tends to argue against a single role for HHV-6 in all of these pathological conditions, the fact that the highest prevalence and the highest mean copy number are found reproducibly by different authors for the nodular sclerosis subtype of HD is quite intriguing. These elements plus the fact that HHV-6 is found mainly essentially in young adults in the case of nodular sclerosis HD may suggest a potential role for HHV-6 in the etiology of HD in young people and simultaneously adds weight to a suspected multiple etiology for HD. Another possibility which must be considered is that immunosuppression occurring during the course of HD, especially concerning T lymphocytes, could induce or provide favorable conditions for the reactivation of an opportunistic virus, such as HHV-6.

In summary, the TaqMan PCR assay developed here provides rapid, reliable, and sensitive quantitative measurement of HHV-6 DNA in saliva and human lymph gland biopsies. The new assay enabled quantitation of increased numbers of HHV-6 DNA genome sequences in the lymph nodes of patients suffering from lymphoproliferative disorders.

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